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Activatable Antibody Mimetics for the Selective Delivery of Therapeutics

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Introduction

Antibodies have become an essential tool not only in basic investigation but particularly in diagnosis and therapy. Nowadays, they are established as therapeutics for several indications, including cancer. Antibody therapeutics are directed against targets overexpressed in diseased cells. However, these targets are rarely expressed only in the diseased tissue and many adverse effects arise from their interaction with the intended target in the healthy tissue [1]. These on-target off-site effects significantly reduce the therapeutic window of many antibody therapeutics. To overcome this issue, conditionally-activated antibodies have been developed. Such antibodies are able to engage their target only upon activation mediated by a specific stimulus [2]. Stimuli-responsive antibodies show an increased therapeutic window compared to their non-activatable counterpart and allow targeting of receptors previously considered undruggable. A wide variety of cues can be harnessed to activate antibodies, including internal stimuli, such as enzymes, pH and ion concentration, as well as external ones, such as light. In the context of cancer therapy, the use of protease- or pH-responsive antibodies seems promising, since they rely on the peculiar characteristics of most tumor microenvironments. Among the many inactivation strategies that have been developed, those that rely on steric hindrance are of particular interest. Despite usually showing smaller differences in binding between active and inactive conformation compared to Probodies, which rely on *N*-terminal epitope mimetic extension, methods relying on steric hindrance can be more easily transferred across different antibody specificities and formats.

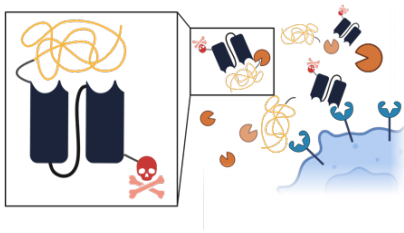

Fig. 1. Schematic representation of the objective of this work.

In our group we are developing new methods to reversibly mask antibodies with high transferability across antibody formats and specificities. Masking moieties used to provide sufficient hindrance with current approaches need to be very large, like in the case of XTENylated antibodies, where *N*-terminal elongations of 600-900 residues are required [3]. This increases considerably the size of the therapeutic, further limiting its diffusion into the tumor tissue. Here we report a new method that enables masking of a single chain variable fragment (scFv) using a protease-cleavable sterically hindering masking moiety of reduced size (Figure 1).

Results and Discussion

scFvs are antibody fragments whose tertiary structure is stabilized by two disulphide bridges. Therefore, their expression in soluble form in bacteria can prove quite challenging, due to the reducing environment in the bacterial cytoplasm. Our scFv was expressed in *E. coli* BL21(DE3) cells in an insoluble form, purified from the inclusion bodies by affinity chromatography, and finally refolded into its active structure by dialysis. The refolded scFv showed the expected mass both on SDS-PAGE analysis and MALDI mass spectrometry. After refolding, the scFv was able to bind specifically to HeLa cells transfected with its antigen, with an apparent K_d in the subnanomolar range (Figure 2).

Once we confirmed that the refolded scFv was active, we mutated residues that were either in more conserved loops around the CDRs or in the CDR themselves into cysteines. These cysteine residues were used as anchor points to site-specifically conjugate peptides and polymers of different sizes via thia-Michael addition. We are currently implementing other strategies we have previously developed based on bioorthogonal chemistry [4,5].

In most of the strategies relying on steric hindrance, such as XTENylation, the masking moiety is expressed with the antibody as an *N*-terminal-extension. Despite the fact that the *N*-terminus is relatively close to the CDRs in the tertiary structure, it is also usually more flexible. This could explain why such big masks are needed to achieve sufficient inactivation. We reasoned that conjugating the masking moiety to more strategically located residues would enable reducing the size of the mask. Successful conjugation was confirmed by Western Blot and LC-MS. The scFv-mask conjugates were assayed in cell binding experiments. We observed apparent K_ds up to 40-fold lower compared to the WT antibody, depending on the site modified and the conjugated mask. Such masks may be removed by tumor-specific proteases.

In parallel, we developed a strategy to generate scFv-drug conjguates by conjugating toxic payloads to this scFv. In particular, we aimed to conjugate the drug at the *C*-terminus of the scFv after introducing a bioorthogonal chemical handle via enzymatic ligation mediated by Sortase A. The transpeptidase was produced in *E. coli* BL21(DE3) cells and purified by affinity chromatography. The ligation reaction was optimized and product formation was observed both with SDS-PAGE (Figure 3) and LC-MS. Conjugates were produced by reacting the bicyclo[6.1.0]non-4-yne (BCN) handle installed on the scFv with tetrazine-functionalized payloads, including toxic molecules and protease-resistant peptides that enable transport across biological barriers [6].

We have shown that our antibody mimetic has high selectivity for target cells. We expect this will translate into reduced on-target off-site effects and, thus, higher selectivity compared to common ADCs in the *in vivo* experiments we will conduct.

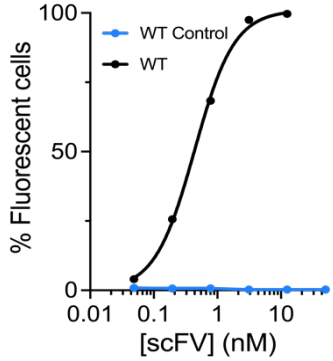

Fig. 2. Binding of refolded scFv on HeLa cells transfected with its target receptor.

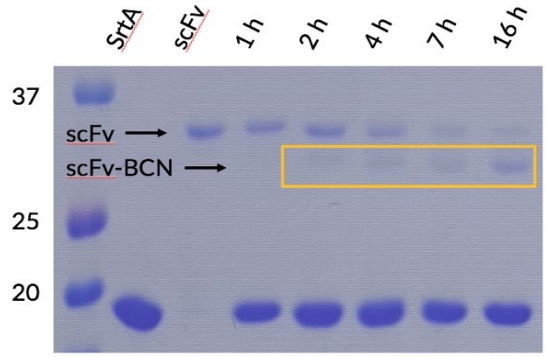

Fig. 3. SDS-PAGE analysis of Sortase A reaction over time.

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